

Developmental Toxicity and Structure/Activity Correlates of Glycols and Glycol Ethers

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In recent years, the National Toxicology Program (NTP) has selected numerous glycol ethers for testing in routine laboratory mammals to ascertain the magnitude of their ability to injure the conceptus. From the lists available of ongoing and projected NTP test chemicals, a series of glycol ethers was selected for examination *in vitro* in the hydra assay. Also tested were additional chemicals of similar molecular configuration and/or composition. This short-term screening test placed the 14 glycols and glycol ethers tested into a rank order sequence according to their degree of hazard potential to developmental biology, i.e., their ability to interfere with the developmental events characteristic of all ontogenic systems. They were ranked according to the difference between the lowest dose or concentration overtly toxic to adults (*A*) and the lowest concentration interfering with development (*D*) of the artificial embryo of reaggregated adult hydra cells and the *A/D* ratio.

Published data from mammalian studies were available for a few of the test chemicals, and in each instance the hydra assay was in direct agreement with the outcomes reported of the more elaborate and standard animal tests. Ethylene glycol and ethylene glycol monomethyl ether were shown by both standard evaluations in mammals, and by the hydra assay, to disrupt embryos only at or very near to their respective adult toxic doses, whereas the mono-ethyl ether perturbed development at approximately one-fifth of the lowest dose overtly toxic to adults.

Data from the hydra prescreen predict that, when tested by the NTP or other sponsor in the standard protocols employing mammals, the majority of the additional glycols and glycol ethers tested in hydra will be found capable of disrupting embryonic development only at, or very near to, exposure levels also overtly toxic to adults treated by the same route of administration. That is, they actually are not high priority items for additional testing provided human exposures are not anticipated at or near their respective adult toxic dose levels.

No obvious structure activity relationship between these molecules and their developmental toxicity was found in the group assayed in hydra.

The glycol ethers are a class of substances widely used in contemporary society. For this and other cogent reasons they are the subject of considerable interest regarding their possible toxicity. Most of the more usual members have been at least partially tested regarding their acute and perhaps target organ toxicity, but very few have been examined for their ability to disrupt *in utero* development. Recently, however, several members of this chemical class have been evaluated in standard assays to determine their potential to disrupt the conceptus and The National Toxicology Program has tested or has cited a larger list of glycol ethers as high priority items for developmental toxicity evaluations in the near future (1). Such tests are time-consuming, require large numbers of pregnant animals, are very expensive and require elaborate facilities and technical expertise of limited availability. Furthermore, and more

relevant to this report, is the fact that the means presently available to select candidates for such definitive studies are imprecise and, of necessity, represent educated guesses at best because until now we have lacked a nonambiguous and biologically relevant screening system to quantifiably prioritize previously untested substances regarding their hazard potential for the conceptus.

During the past several years our laboratory has been developing (2,3) and then documenting (4,5) the ability and reliability of an *in vitro* developmental toxicity screening system to predict and recapitulate experimental findings of rigorously executed developmental toxicity evaluations made by state-of-the-art protocols in the more standard laboratory animals. The hydra assay demonstrated a remarkable ability to recapitulate the data of complex studies made in pregnant mammals, and it began to be used by private corporations prospectively to evaluate previously untested substances. In this vein of prospective testing to identify substances

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uniquely hazardous to developmental biology and differentiate them in a quantified or prioritized manner from substances capable of injuring embryos only at exposure levels so high that they were overtly toxic to adults, we tested several members of this chemical class. For the present study, a number of glycols and glycol ethers were selected from the list of such chemicals designated for developmental toxicity testing by the NTP. These were evaluated for this parameter in the hydra assay. Those selected were chosen, and supplemented with additional glycols, on the basis of their molecular structure in an attempt to reveal any possible structure/activity relationships between one or more of these molecule's configuration or composition and its hazard potential for disrupting any of the developmental biologic events so characteristic of any and all ontogenic systems.

Materials and Methods

Adult polyps of the fresh water coelenterate *Hydra attenuata* were grown in a specially prepared apparatus where they would reproduce by asexual budding and could be fed and cleaned of expelled detritus on a regular schedule with a minimum of technical effort (4). To begin each assay, approximately 700 to 1000 clean adult hydra (Fig. 1A) were collected and then dissociated mechanically into their component cells which were then randomly reassociated by being gently packed into small pellets (Fig. 1B) through low-speed centrifugation (2). Previous experimentation has established that these randomly reaggregated cells will undergo a full ontogenesis sequence and give rise to a new population of adult polyps in about 92 hr if they are left undisturbed (Fig. 1C). These artificial preparations consist of two broad classes of cells (6). The greater number by far are fully differentiated adult cells which quickly achieve spatial orientation and migrate to a position consistent with their phenotype. That is, endoderm cells of the original polyps become positioned internally within the aggregate whereas the ectoderm cells migrate to the periphery where they then form normal intercellular membranous contacts (E. M. Johnson, unpublished data) and begin to elaborate extracellular matrix in the form of a new basement membrane. The second broad class of cells consists of a much smaller number of undifferentiated interstitial cells capable of becoming any of the numerous differentiated cells typical of adult hydra. It is these interstitial cells which undergo rapid and markedly localized proliferation and, by means as yet undefined, are induced, in sharply localized regions, to differentiate into tentacles and the exquisitely elaborate nematocysts so characteristic of these simple yet highly organized animals. During their >90-hr period of differentiation and organogenesis, these artificially manufactured pellets achieve all of the developmental biologic phenomena

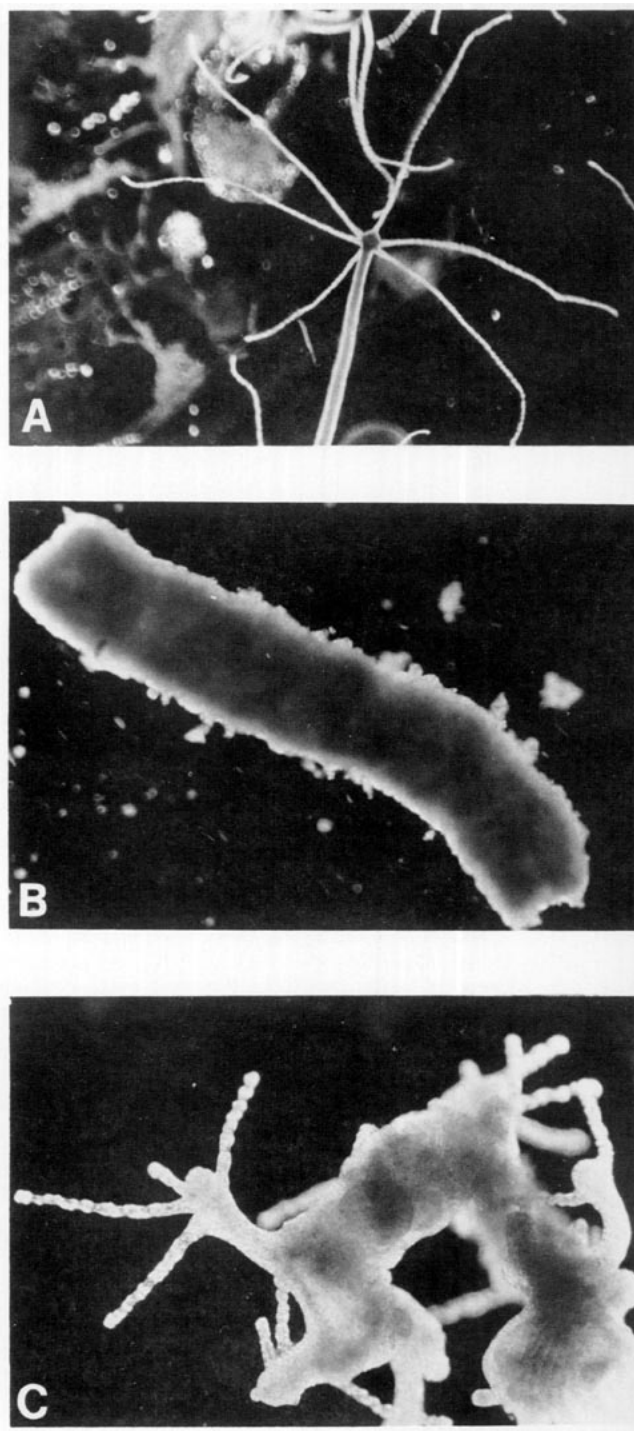


FIGURE 1. Micrographs showing (A) the apical end of a fully extended adult hydra showing the elevated hypostome and surrounding tentacles. The entire animal is about 1 cm tall. (B) One of about 20 pellets (artificial "embryo") derived from repacking the dissociated cells of 700 to 1000 adult hydra. It has begun differentiation as evidenced by the partially smoothed external surface. This preparation is approximately 1 cm in length. (C) An artificial "embryo" after about 92 hr of differentiation and organogenesis. Approximately 10 to 20 adults will form simultaneously from one pellet and eventually will detach to form free-standing polyps.

known to occur in an embryo of any species (7), and in view of this fact, they are termed artificial "embryos."

The testing protocol used in each evaluation was not varied from one chemical to another and no prior information was needed in order to test any substance. All test chemicals were water-soluble except ethylene glycol monophenyl ether and diethylene glycol dibutyl ether. These were diluted using ethanol as a general procedure in instances where insolubility is observed with test chemical concentrations below 10 mL/L. The protocol employed in this study was a three-step procedure somewhat shortened from previous reports. It was used twice for each chemical tested; once for "embryos" and once for adult hydra. Step 1 employs test chemical concentrations at whole-log concentration intervals from 1×10^{-3} through 1×10^3 mL/L. This step identifies the lowest whole-log concentration of the chemical capable of disrupting development of the embryo and when repeated with adult animals, the lowest concentration capable of producing the adult endpoint sign of toxicity. The second step of the testing protocol for both embryos and adults again tests the lowest toxic whole-log concentration eliciting the toxic endpoints obtained in the first step as well as the next lowest whole-log concentration, i.e., the highest whole-log no-effect level. The main part of step 2 is to test the eight 1/10 log concentrations between these two whole-log concentrations. For example, assume that in step 1 concentrations of 10^3 and 10^2 mL/L caused the endpoint, but a concentration of 10^1 mL/L did not. In step 2, 10^2 mL/L, the lowest whole-log concentration eliciting the toxic endpoint would be rerun as would the next lowest whole-log concentration of 10^1 . One thereby has bracketed the as-yet-undetermined 1/10 log dose. There are eight 1/10 log concentrations between 10^2 and 10^1 , and these eight

concentrations were also run, i.e., the concentrations tested here would be: 10^2 or 100, 90, 80, 70, 60, 50, 40, 30, 20 and 10^1 or 10 mL/L. By this protocol, this step determines to within 1/10 log those minimal effective concentrations (MEC) of the test substance capable of producing adult and developmental toxicity. The final step is a confirmation of the MEC observed in step 2. The adult MEC (A) and the developmental MEC (D) are calculated as a ratio. This A/D ratio is considered as a developmental toxicity hazard index whose increasing size is directly proportional to a chemical's ability to injure embryos in the absence of adult toxicity. The chemicals tested are listed in Table 1.

Results

The 14 glycols and glycol ethers tested in this short study are listed in Table 1 alone with both the adult and the developmental MEC and their A/D ratios. The ratios varied from 1.0 through 5.0 with diethylene glycol and the three acetates being the lowest. These four chemicals proved capable of disrupting development only at concentrations which were also toxic to the adult animal; the minimal effective (toxic) concentration or MEC for adult and for developmental toxicity were the same, e.g., 30 mL/L for each in the case of diethylene glycol. Each of the three acetate forms tested were toxic at rather low exposure concentrations, but each injured the adults and "embryos" at the same concentration. The A/D ratio for ethylene glycol monoethyl ether was 5.0, and that of ethylene glycol monobutyl ether was 4.4. These were the highest in this group of chemicals. It required 30 mL/L to produce the toxic endpoint in adults, yet the "embryo's" development was disrupted by only 6.0 mL in the case of the monoethyl ether, but

Table 1. Developmental toxicity hazard potential of glycols and glycol ethers.

Test chemical (CAS #)	A = adult MEC ^a	D = "embryo" MEC ^b	A/D ratio ^c
Ethylene glycol (102-21-1)	50.0	30.0	1.7
Propylene glycol (57-55-6)	40.0	30.0	1.3
Hexylene glycol (107-41-5)	20.0	6.0	3.3
Ethylene glycol monomethyl ether (109-86-4)	40.0	30.0	1.3
Ethylene glycol monoethyl ether (111-15-9)	30.0	6.0	5.0
Ethylene glycol monobutyl ether (111-76-2)	4.0	0.9	4.4
Ethylene glycol monophenyl ether (122-99-6)	1.0	0.3	3.3
Ethylene glycol monomethyl ether monoacetate (110-49-6)	0.7	0.7	1.0
Ethylene glycol monoethyl ether monoacetate (111-15-9)	0.6	0.6	1.0
Ethylene glycol diacetate (111-55-7)	0.2	0.2	1.0
Diethylene glycol (111-46-6)	30.0	30.0	1.0
Diethylene glycol monomethyl ether (111-77-3)	30.0	20.0	1.5
Diethylene glycol monoethyl ether (111-90-0)	20.0	9.0	2.9
Diethylene glycol dibutyl ether (112-73-2)	0.9	0.4	2.2

^aThat lowest concentration (to within 1/10 log) of the test chemical capable of producing the sign of toxicity in adult hydra.

^bMEC for development of the artificial "embryo."

^cThe decimal points are retained for the sake of arithmetic accuracy. Such a degree of biologic precision is not a possibility in any developmental toxicology safety evaluation or in this screening assay.

the monobutyl ether achieved toxic effects at much lower exposures. The addition of an acetate group to the monoethyl ether markedly altered the *A/D* ratio (reduced it from 5 to 1.0). Ethylene glycol monoethyl ether monoacetate was coeffective, i.e., both the adult and the "embryo" were injured at the same concentration. Ethylene glycol monophenyl ether and hexylene glycol had the second highest *A/D* ratios (3.3), and the monophenol was also the second most toxic of the substances tested from the point of developmental toxicity. Only 0.3 mL/L was needed to cause an effect, and the only chemical tested here which was toxic at a lower exposure level was ethylene glycol diacetate.

There appeared to be no relationship between a chemical's hazard potential to the embryo (*A/D* ratio) and its general toxicity, i.e., lowest exposure level capable to producing toxicity in this system; though all three of the acetate forms tested were coeffective and obviously have no propensity to injure developmental biology. Also absent is any obvious correlation between molecular size or composition and either general toxicity or propensity to disrupt the developmental sequences of the embryo. Each of the chemicals tested seemed to disrupt a different phase or portion of the "embryo" on slightly different sequences of time or dose response, but such is not directly relevant to the present discussion. To be complete, it should be pointed out that though the *A/D* ratio proves predictive of the ratio in mammals, the exposure levels needed to produce toxicity in hydra can be quite different from those in mammals, where metabolism, binding or dissociation may occur.

Discussion

Any substance administered to a pregnant female will injure the conceptus if the dose or exposure level is high enough. As reluctant as some of us may be to accept this basic fact, experimental findings cannot be ignored. To be of practical utility, this concept must be placed into a realistic perspective (8,9) consistent with knowledge acquired by developmental biologists over several centuries (10). This concept must be considered in conjunction with a second basic understanding. There are four manifestations of perturbed embryonic development: death of the products, developmental delays (runts), terata, and decrements of normally expected functional attainment levels (11). Each of these is an unacceptable developmental outcome if dose-related to any specific agent of either voluntary or involuntary exposure. Once these two concepts are appreciated, it becomes possible to remove developmental toxicology from the black box of a binary designation: teratogen vs. nonteratogen, minor variation vs. abnormality, embryotoxic vs. teratogenic, and one finds that quantitative risk estimation based on standard safety factors is possible.

The spectrum of adult/developmental toxicity seen here from data of hydra and glycol ethers is identical to the answers of the same question posed for glycols as well as innumerable other chemicals in relation to

hazard potential for development. For instance, aspirin administered to pregnant animals will not perturb embryonic development until the dose level approaches very near to that also toxic to the adult; its *A/D* ratio is about 2. That for benzene is less than one, indicating a degree of maternal protection, if you will—or at least, that developmental events of the conceptus are less vulnerable to its action than is the adult animal. The method by which the hydra assay achieves its relevance as a screening system is that it establishes this relationship at a fraction of the cost in time and resources as rats and rabbits. The regulatory and safety evaluation scene in developmental toxicology is confused by application of concepts and terms appropriate for mutagenesis and carcinogenesis but inappropriate and actually counterproductive of embryonic safety when applied to the myriad of known and unknown phenomena essential for normal development to occur in the mammalian conceptus (11) or the hydra "embryo" (7). One must not confuse teratology with developmental toxicity. It is but one of its manifestations and is no more or no less acceptable than any of the other three ways in which a developing system can exhibit injury. It is unfortunate that we refer to the most common developmental toxicity test (the segment II evaluation in rats and rabbits) as the teratology test. Actually, of course, it is not designed to produce terata and would be of markedly different design if it were. It is designed to assess the toxic effects of substances on the total developmental biology of the conceptus and always in relationship to adult toxicity and is more apt to kill embryos than render them abnormal but alive. Thalidomide, aspirin, table salt and vitamin A are all toxic to embryos, but only one is a hazard to development under normal exposure—thalidomide—and this is because it has an *A/D* ratio in the neighborhood of 60 (depending on how one accounts for solubility in the published literature on the topic, it could be calculated somewhat higher or lower). Each of the others is no hazard to embryos as long as the dose is not high enough to injure the mother. Should they be used at so high an exposure level, then surely they too would damage the embryo (12). The hydra assay quickly sorts out the substances worthy of closer developmental toxicity examination in elaborate systems while clearing the reputations of the great majority while also simultaneously providing a quantified and objective rank order for each. The hydra assay, therefore, allows us to focus attention on substances with a predilection to disrupt development and these become prime candidates for more definitive evaluations in real embryos, so the no-observed effect level can be determined prior to cross-species extrapolation after testing in mammals (13,14). The coeffective substances merit no further testing in pregnant animals as the NOEL can be extrapolated based on adult toxicity endpoint assays.

The results of these hydra assays of glycols and glycol ethers typify results to be expected in mammals. The great majority of substances do not merit elaborate and

time consuming studies of developmental toxicity. They are not good candidates for elaborate and expensive tests unless exposure expectations indicate women will be exposed to near adult toxic levels. All hydra does is quickly identify this majority while ranking the minority according to their degree of hazard to developmental phenomena. It is excellent for quantitative hazard detection because it predicts the *A/D* ratio. It is irrelevant to quantitative risk estimation, because the doses in hydra are often quite different from those needed to produce mammalian toxicity. None of the glycols or glycol ethers tested has a marked predilection for the embryo. Most disrupt development only at or near adult toxic exposure levels, but a few have some modest ability to perturb developmental biology at a fraction of the concentration toxic to adults.

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The hydra assay is patented by Thomas Jefferson University (U.S. Patent No. 4346070), and unrestricted licensing is available through Mr. Mark Herrmann, Director of Business Services, Scott Building, Thomas Jefferson University, 1025 Walnut Street, Philadelphia, PA 19107.

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